(IB; 1:1,000, BD Transduction Lab.), anti-Xpress (IP/IF: 1:500; IB: 1:5,000, Invitrogen), anti-Flag (IP: 1:500; IB: 1:2,000, Sigma), anti-HDAC-1 (IB: 1:1,000), anti-E2F1 (IB: 1:500, Santa Cruz), anti-α-tubulin (IB: 1:1,000), anti-β-actin (IB: 1:1,000, Sigma), anti-p21 (IB: 1:500, Pharmingen), anti-p15 (IB: 1:500, Santa Cruz) and anti-EEA1 (IF:1:250, BD Transduction Lab.).

Apoptosis, cell growth and senescence assay

B cells isolated from 8–12-week-old wild-type and *Pml*$^{-/-}$ mice were treated with vehicle or TGF-β1 for 30 h. Cells were fixed, and the TUNEL assay was performed using the *in situ* cell death detection kit (Roche) according to the manufacturer's instructions. For growth assays, after TGF-β1 treatment, cells were harvested and stained by trypan blue at different days of TGF-β1 treatment, and numbers of viable cells were directly counted by light microscopy. A thymidine incorporation assay was performed as previously described[29]. For senescence assays, wild-type or *Pml*$^{-/-}$ MEFs were either left untreated or treated with TGF-β1 for 6 days, and cells were fixed and stained with β-galactosidase (β-gal) using the senescence detection kit (Oncogene).

Sucrose flotation gradient

Early-endosome-containing fractions were prepared as described[30], with some modifications. In brief, MEFs (6–8 near-confluent 10-mm dishes) cultures were washed twice with ice-cold PBS, scraped in PBS and centrifuged at 1,200 *g*. After addition of 5 ml homogenization buffer (250 mM sucrose, 3 mM imidazole at pH 7.4, 0.5 mM EDTA), cell pellets were put on ice for 5 min, centrifuged and resuspended in 0.5 ml homogenization buffer containing the protease inhibitor cocktail (Roche). Cells were then homogenized at 4 °C by seven passages through a 25G 5/8 needle fitted onto a 1 ml plastic syringe. Homogenates were centrifuged for 10 min at 2,000 *g* at 4 °C and the post-nuclear supernatants (PNSs) were collected. The PNS fraction was then brought to 40.6% sucrose using a stock solution (62% sucrose, 3 mM imidazole at pH 7.4) (final volume 1.1 ml) and loaded at the bottom of an SW 60 centrifugation tube. A gradient consisting of three steps was then poured (1.5 ml of 35% sucrose, 3 mM imidazole at pH 7.4; 1 ml 30% sucrose, 3 mM imidazole at pH 7.4; 0.5 ml homogenization buffer). The gradient was centrifuged at 485,000 *g* for 90 min using an SW60 rotor. Eight 0.5 ml fractions were collected from the top of the tube, and a portion (40 μl) of each was subjected to SDS–PAGE analysis, followed by western blot analysis.

Transcriptional assays

MEFs and HepG2 cells were transfected by using superfect (Qiagen) for 3 h, and cells were treated with vehicle or 100 pM TGF-β1 for 30 h. The luciferase activity was measured by using the dual luciferase system (Promega). The results were normalized over renilla activity.

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Correspondence and requests for materials should be addressed to P.P.P. (p-pandolfi@ski.mskcc.org).

Induction of DNA methylation and gene silencing by short interfering RNAs in human cells

Hiroaki Kawasaki & Kazunari Taira

Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan, and Gene Function Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba Science City 305-8562, Japan

Double-stranded RNAs (dsRNAs) induce post-transcriptional gene silencing in several species of animal and plant[1–2]. In plants, dsRNAs targeted to CpG islands within a promoter can also induce RNA-directed DNA methylation[3–8]; however, it remains unclear whether gene silencing mediated by DNA methylation can be induced by dsRNAs in mammalian cells. Here, we demonstrate that short interfering RNAs (siRNAs; 21–25-nucleotide RNA molecules) induce DNA methylation and histone H3 methylation in human cells. Synthetic siRNAs targeted to CpG islands of an E-cadherin promoter induced significant DNA methylation and histone H3 lysine 9 methylation in both MCF-7 and normal mammary epithelial cells. As a result, these siRNAs repressed expression of the E-cadherin gene at the transcriptional level. In addition, disrupting the expression of either one of two DNA methyltransferases (*DNMT1* or *DNMT3B*) by specific siRNAs abolished the siRNA-mediated methylation of DNA. Moreover, vector-based siRNAs targeted to the *erbB2* (also known as *HER2*) promoter also induced DNA methylation in MCF-7 cells. Thus, siRNAs targeted to CpG

islands within the promoter of a specific gene can induce transcriptional gene silencing by means of DNA-methyltransferase-dependent methylation of DNA in human cells, and might have potential as a new type of gene therapeutic agent.

Double-stranded RNAs induce RNA interference (RNAi)-mediated post-transcriptional gene silencing in animals and plants[1,2]. In this system, siRNAs are generated by the RNase III Dicer enzyme and are incorporated into the RNAi-induced silencing complex (RISC)[9,10]. The siRNA–RISC complex then promotes degradation of cytoplasmic messenger RNAs[11–16].

To determine whether synthetic siRNAs might induce DNA methylation in mammalian cells, we synthesized siRNAs targeted to CpG islands of the E-cadherin promoter (E-cadherin-siRNAs), as E-cadherin can be silenced by aberrant methylation of the promoter in several lines of tumour cells[17–19]. Moreover, as CpG sites in the E-cadherin promoter are unmethylated in MCF-7 cells[17,18], we chose MCF-7 cells for this study. We selected ten CpG sites (sites 1–10) in the E-cadherin promoter as targets of siRNAs (Fig. 1a). Each E-cadherin-siRNA (100 nM) was introduced into MCF-7 cells and, 96 h later, total DNA was collected and genomic DNA was isolated. We then performed bisulphite sequencing to determine the methylation status of the E-cadherin promoter. We treated genomic DNA with bisulphite using a CpGenome DNA-modification kit (Intergen). We amplified the bisulphite-modified E-cadherin promoter by polymerase chain reaction (PCR) and cloned the product of PCR using a TA cloning kit (Clontech). We picked ten independent colonies for each target site and analysed the cloned sequences by direct sequencing.

As shown in Fig. 1b, we found methylated DNA in MCF-7 cells that harboured each respective E-cadherin-siRNA (sites 1–10) and cells harbouring all E-cadherin-siRNAs (sites 1–10; the number of grey squares indicates the number of methylated cytosines). By contrast, a mutant form of the E-cadherin-siRNA directed against site 1 that had eight point mutations in both its sense and antisense strand failed to induce methylation. Moreover, a combination of all E-cadherin-siRNAs together failed to induce DNA methylation within a non-targeted *erbB2* promoter (Supplementary Fig. S1), highlighting the specificity of the E-cadherin-siRNAs. We next examined the possibility of methylation beyond the target site in the absence of RNA-directed RNA polymerase (RdRP) in mammalian cells. Notably, siRNAs targeted to site 3 of the E-cadherin promoter induced methylation not only at that site but also at the adjacent site (site 2), at least to some extent (Fig. 1c). However, site 1, located ~200 nucleotides upstream of site 3, was unmethylated.

Aberrant methylation is often detected in cancer cells. Therefore, we next examined the induction of DNA methylation by siRNAs in

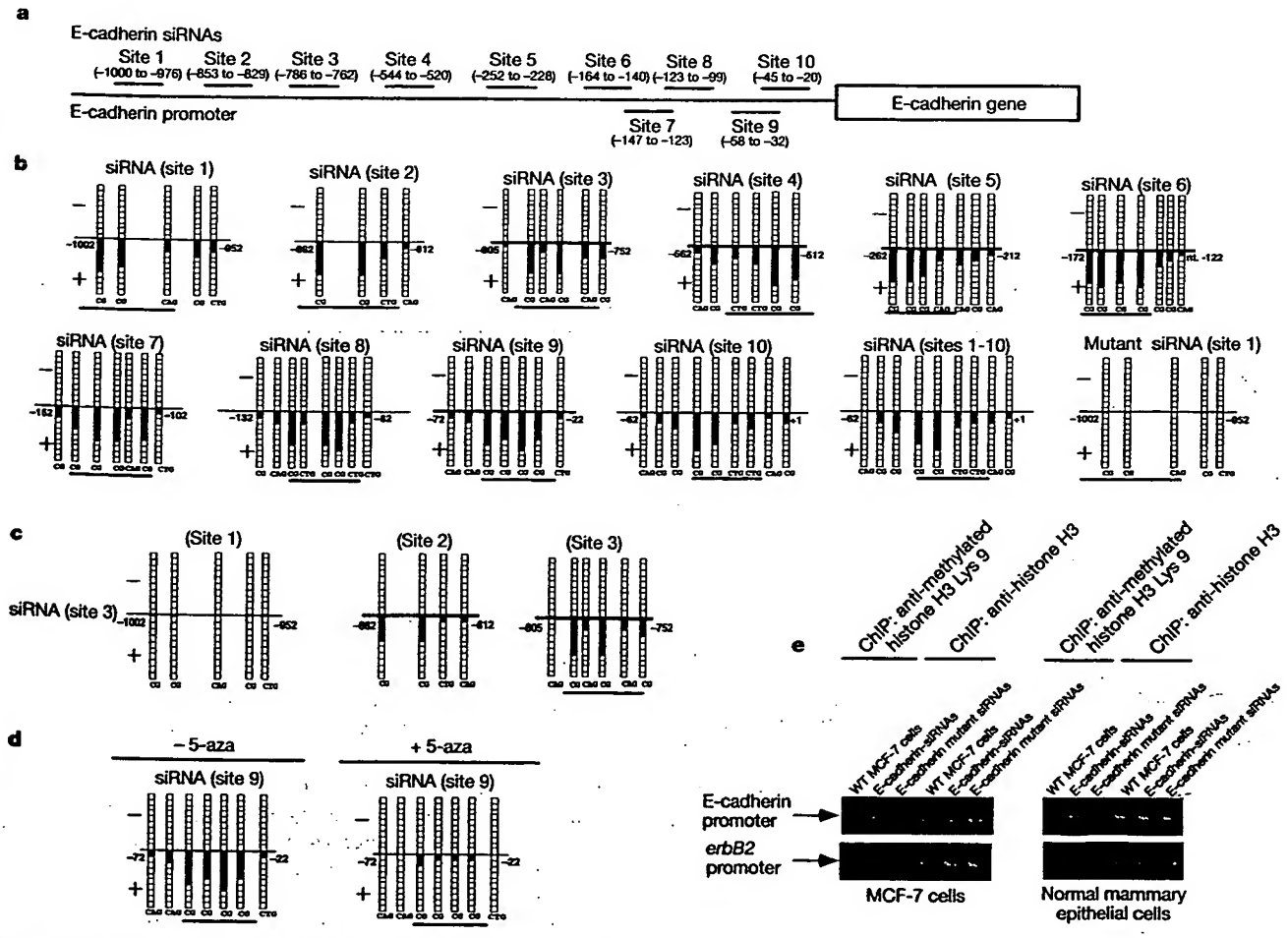

Figure 1 DNA methylation of the E-cadherin promoter by siRNAs in MCF-7 and normal breast epithelial cells. **a**, The ten target sites for siRNAs in the E-cadherin promoter. Nucleotides are indicated in parentheses. **b**, Induction of DNA methylation within the E-cadherin promoter in MCF7 cells by siRNAs, as determined by bisulphite sequencing (see text for details). The number (out of a possible ten) and position of methylated cytosines are indicated by grey squares; underlining indicates the target sequences of siRNAs. **c**, Details of extended methylated regions within the E-cadherin promoter when siRNA was targeted to site 3 within the E-cadherin promoter in MCF7 cells, as determined by bisulphite sequencing. **d**, Relative levels of DNA methylation of the E-cadherin promoter at site 9 in MCF7 cells in the presence or absence of 5-aza, as determined by bisulphite sequencing. **e**, Detection of methylated histone associated with the E-cadherin promoter in cells that were treated with a combination of E-cadherin siRNAs (site 1–10). Methylated histone H3 at lysine 9 was detected using a chromatin immunoprecipitation (ChIP) assay with specific antibodies for methylated histone H3 at lysine 9. Histone H3 antibody is a positive control. WT, wild type.

normal breast epithelial cells. We detected methylation of CpG sites in normal breast epithelial cells that had been treated with E-cadherin-siRNAs (testing methylation at sites 6 and 10; Supplementary Fig. S2). Furthermore, the extent of methylation in MCF-7 cells that had been treated with a combination of all ten E-cadherin-siRNAs was reduced in the presence of 1 μM 5-aza-2′-deoxycytidine (5-aza), an inhibitor of DNA methylation (Fig. 1d).

As methylation of histone H3 at lysine 9 is induced by RNAi in plants, fission yeast and *Drosphila*[8,20,21], we examined whether our set of siRNAs could induce its methylation in mammalian cells using a chromatin immunoprecipitation assay with specific antibodies for methylated histone H3 at lysine 9. Each cell lysate from MCF-7 cells and normal mammary epithelial cells in the presence or absence of the E-cadherin-siRNAs (sites 1–10) was incubated with specific antibodies for methylated histone H3 at lysine 9 or antibodies for histone H3, and the DNA interacting with the methylated histone H3 was immunoprecipitated. The precipitated DNA was then amplified using specific primers for the E-cadherin promoter. As shown in Fig. 1e, the precipitated DNA from the cell lysate of E-cadherin-siRNA-treated MCF-7 cells produced a band corresponding to the E-cadherin promoter region. By contrast, we did not detect the corresponding band when the same procedure was performed in the absence of E-cadherin-siRNAs or in the presence of mutant siRNAs. Similar results were obtained using cell lysate from normal mammary epithelial cells. In addition, it has been reported that an RNA component is involved in maintenance or stabilization of a higher-order structure at pericentric heterochromatin in mammalian cells[22].

Taken together, these results suggest that siRNAs targeted to the E-cadherin promoter can induce not only DNA methylation but also histone H3 methylation at lysine 9 in human cancer cells and normal cells, and that the effects of siRNAs can spread to nearby regions even in the absence of RdRP.

To examine whether the induction of DNA methylation by E-cadherin-siRNAs was correlated with expression of the E-cadherin gene, we performed northern blotting analysis with a probe specific for E-cadherin mRNA. As shown in Fig. 2a, the level of E-cadherin mRNA expression in each line of MCF-7 cells harbouring a specific E-cadherin-siRNA was lower than in wild-type MCF-7 cells (lanes 1–11). Notably, the level of E-cadherin mRNA expression in MCF-7 cells that had been treated with a combination of all E-cadherin-siRNAs (lane 12) was significantly lower than in MCF-7 cells transfected with a single siRNA, clearly demonstrating the additive effects of the siRNAs. Treatment with 5-aza almost completely abolished the effect of the E-cadherin-siRNAs (Fig. 2b, lane 4). Western blot analysis with an E-cadherin-specific antibody also revealed similar reductions in the amount of E-cadherin (Fig. 2c). Thus, siRNAs targeted to the E-cadherin promoter act as gene silencers at the transcriptional level, and induction by siRNAs of DNA methylation within the E-cadherin promoter is inversely correlated with the level of gene expression and is additive.

DNA methyltransferases (DNMTs) are responsible for all DNA methylation in the cell[23–25]. To determine whether DNMTs participate in siRNA-mediated DNA methylation in human cells, we tried to suppress the expression of genes for DNMTs using siRNAs targeted to the respective mRNAs. We synthesized three separate siRNAs that targeted to *DNMT1*, *DNMT2* and *DNMT3B* mRNAs, respectively. As controls, we used mutant *DNMT1*-, *DNMT2*- and *DNMT3B*-siRNAs with four point mutations in both the sense and antisense strand. We introduced *DNMT*-siRNA or mutant *DNMT1*-siRNA at 100 nM into MCF-7 cells using Oligofectamine, and examined the amount of DNMTs by western blotting. Amounts of DNMT1, DNMT2 and DNMT3B in MCF-7 cells transfected with *DNMT1*-siRNA, *DNMT2*-siRNA or *DNMT3B*-siRNA, respectively, but not the corresponding mutant *DNMT*-siRNAs, were significantly lower than those in wild-type MCF-7 cells (Fig. 3a).

To examine whether reduced expression of DNMT genes affects siRNA-mediated DNA methylation, we introduced a combination of E-cadherin-siRNAs (sites 1–10) into MCF-7 cells transfected with *DNMT1*-, *DNMT2*- or *DNMT3B*-siRNAs. As shown in Fig. 3b, the extent of DNA methylation induced by E-cadherin-siRNAs in MCF-7 cells transfected with *DNMT1*- and *DNMT3B*-siRNA was significantly lower than in wild-type MCF-7 cells or in MCF-7 cells transfected with mutant *DNMT*-siRNA. By contrast, disruption of

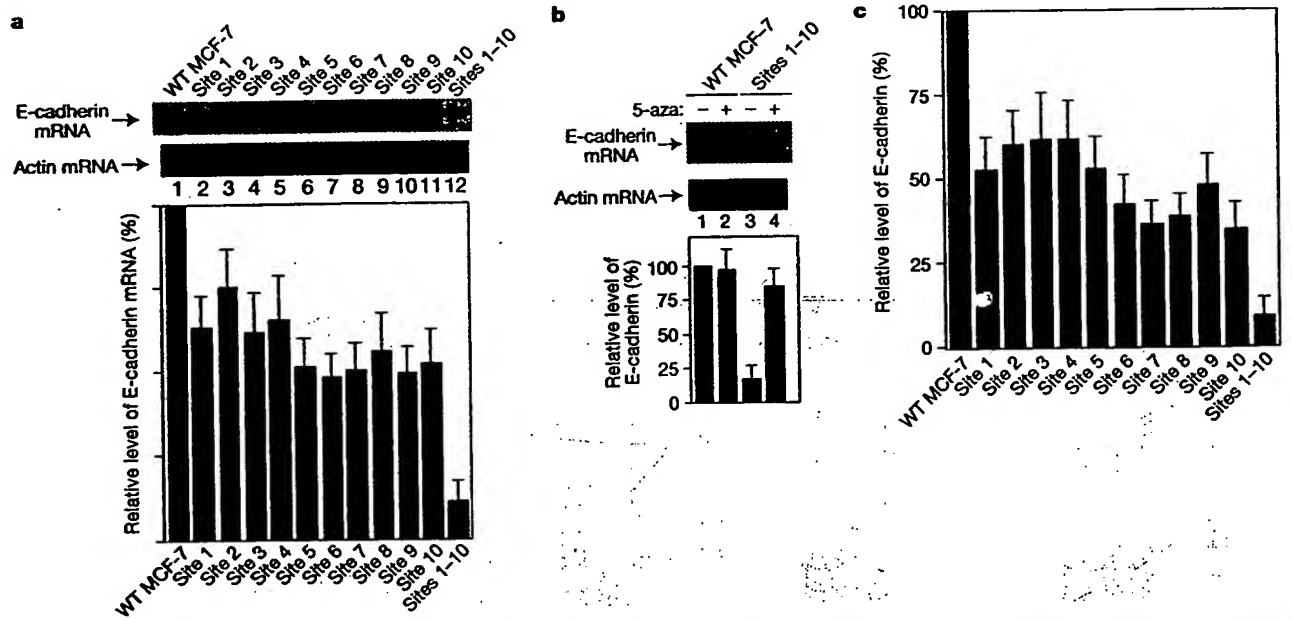

Figure 2 Effects of E-cadherin-siRNAs targeted to the E-cadherin promoter on E-cadherin mRNA expression. **a**, Levels of E-cadherin mRNA expression in cells transfected with E-cadherin-siRNAs. Total RNA from each line was analysed by northern blotting analysis. Actin mRNA was used as the endogenous control. Values are means ± s.d. **b**, Relative levels of E-cadherin mRNA expression in cells transfected with E-cadherin-siRNAs and grown with and without 5-aza. **c**, Levels of E-cadherin expression in cells transfected with E-cadherin-siRNAs. E-cadherin in each line was detected by western blotting with specific antibodies. Relative levels of E-cadherin were analysed densitometrically. Values are means ± s.d.

DNMT2 expression did not significantly affect siRNA-mediated DNA methylation. Thus, *DNMT1* and *DNMT3B*, but not *DNMT2*, seem to be necessary for siRNA-mediated DNA methylation in human cells.

We next examined the effects of E-cadherin-siRNAs on the expression of E-cadherin in MCF-7 cells transfected with *DNMT*-siRNA. As shown in Fig. 3c, siRNAs targeted to the E-cadherin promoter did not alter E-cadherin gene expression in MCF-7 cells that contained siRNAs targeted to *DNMT1* or *DNMT3B* mRNA (lanes 4 and 16). By contrast, E-cadherin-siRNAs suppressed E-cadherin gene expression in MCF-7 cells that contained *DNMT2*-siRNA or mutant *DNMT*-siRNAs (lanes 6, 10, 12 and 18), demonstrating that *DNMT1* and *DNMT3B* are necessary for the transcriptional gene silencing that results from the induction of DNA methylation by E-cadherin-siRNAs.

To examine the possibility for gene therapy via the control of DNA methylation by siRNAs, we constructed expression vectors for short hairpin RNAs (shRNAs) targeted to an *erbB2* promoter. The *erbB2* gene is overexpressed and unmethylated in several lines of tumour cells, such as MCF-7 cells, whereas it can be silenced by methylation of the promoter in several lines of normal cells[26]. To express shRNAs in cells, we used the well-characterized $tRNA^{Val}$ promoter system[16]. We have demonstrated previously that $tRNA^{Val}$-driven shRNA induces siRNA-mediated gene silencing in human cells[16]. We selected five CpG islands (sites 1–5) within the *erbB2* promoter as targets of shRNAs. Then, we introduced these shRNA expression plasmids transiently into MCF-7 cells. We confirmed by means of northern blotting that appropriate processing and production of siRNAs had occurred in cells that expressed tRNA-shRNAs (Fig. 4b, lanes 1–5), and then we examined levels of DNA methylation within the *erbB2* promoter. We isolated genomic DNA from each cell line and performed bisulphite sequencing. As shown in Fig. 4c, we detected DNA methylation within the *erbB2* promoter in all lines of MCF-7 cells that expressed a tRNA-shRNA or a combination of all tRNA-shRNAs (sites 1–5). However, a mutant tRNA-shRNA (site 1) with nine point mutations in both the sense and antisense strand did not induce DNA methylation within the *erbB2* promoter. In addition, tRNA-shRNA did not induce DNA methylation within the non-targeted E-cadherin promoter (Supplementary Fig. S3). We obtained similar results with U6 promoter-driven shRNAs (data not shown), demonstrating that induction of sequence-specific DNA methylation by vector-based siRNAs in human cells is a general phenomenon.

To determine whether the vector-based shRNAs had suppressed the expression of *erbB2*, we examined levels of *erbB2* mRNA expression by means of northern blotting. As shown in Fig. 4d, the expression level of *erbB2* mRNA in cells that expressed each respective tRNA-shRNA was lower than in wild-type MCF-7 cells.

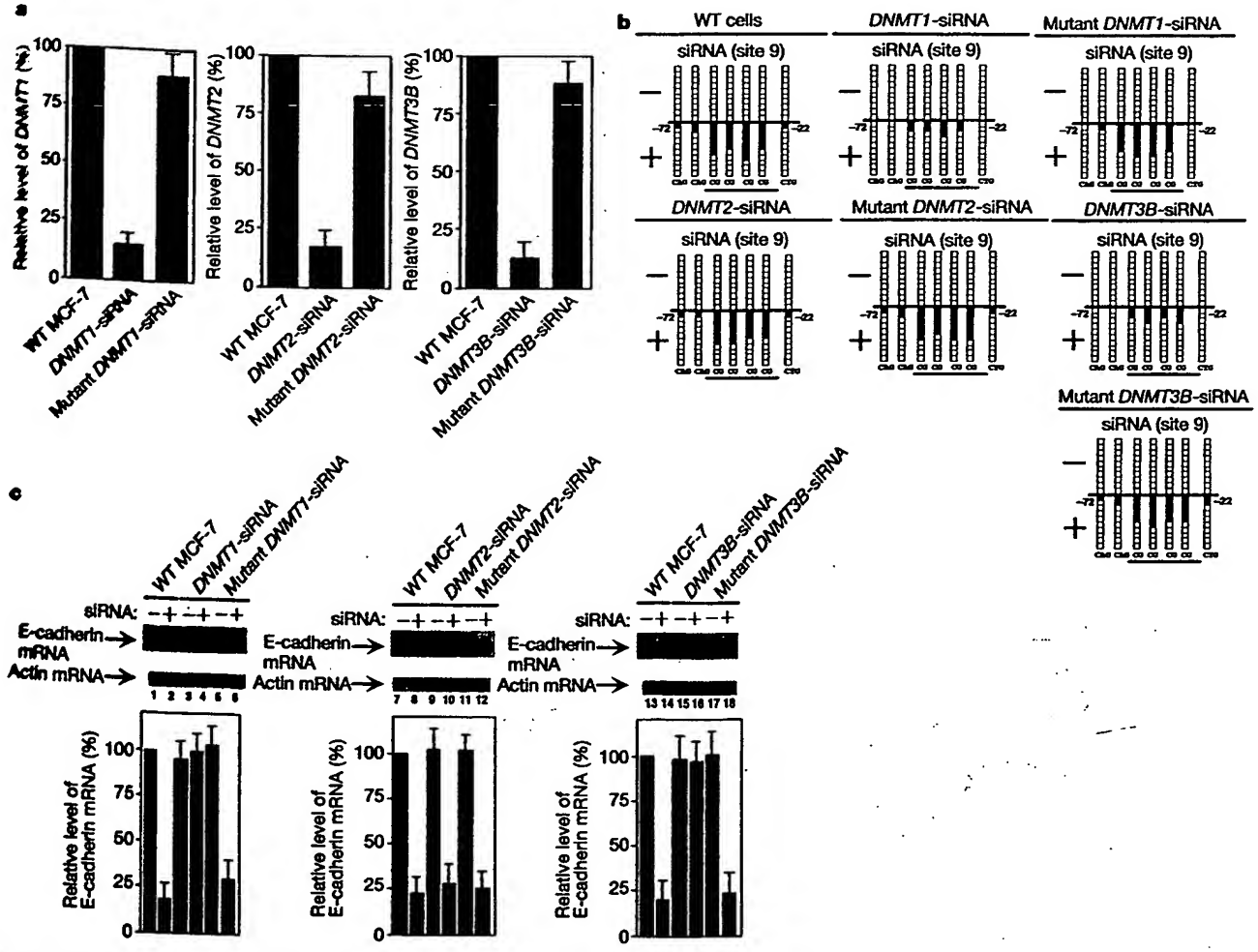

Figure 3 Effects of E-cadherin-siRNAs targeted to the E-cadherin promoter in DNMT1-, DNMT2- and DNMT3B-knockdown cells. **a**, Amount of DNMTs in MCF-7 cells in the presence or absence of *DNMT*-siRNAs. The amount of DNMT1, DNMT2 and DNMT3B was detected by western blotting. Values are means ± s.d. **b**, Level of DNA methylation of the E-cadherin promoter in MCF-7 cells in the presence or absence of E-cadherin-siRNA and various *DNMT*-siRNAs. DNA methylation was detected by bisulphite sequencing. **c**, E-cadherin mRNA expression levels in MCF-7 cells in the presence or absence of E-cadherin-siRNA (sites 1–10) and various *DNMT*-siRNAs. Total RNA from each line was analysed by northern blotting with specific probes. Values are means ± s.d.

As observed previously (Fig. 2a), the level of *erbB2* mRNA expression in cells that expressed all tRNA-shRNAs (sites 1–5) together was significantly lower than in wild-type MCF-7 cells and in cells that expressed individual tRNA-shRNAs, demonstrating the additivity of suppression by vector-based shRNAs at the transcriptional level. Transcriptional regulation through the induction of methylation within a promoter by siRNA in mammalian cells seems to be a general phenomenon, as demonstrated by the examples in this study.

To examine the phenotype of cells that expressed tRNA-shRNAs, we analysed the proliferation rates of various cell lines. MCF-7 cells expressing all tRNA-shRNAs (sites 1–5) proliferated significantly more slowly than wild-type MCF-7 cells and cells that expressed tRNA-shRNAs targeted to a puromycin-resistance gene, which we chose as a nonspecific control (Fig. 4e). The reduced proliferation rate of MCF-7 cells that expressed all tRNA-shRNAs (sites 1–5) was correlated with a reduction in the level of *erbB2* mRNA expression in these cells, suggesting that the tRNA-shRNAs targeted to the *erbB2* promoter might have potential utility as therapeutic agents.

The DNA methylation of promoters has an important role in the genesis and development of tumours by regulating the expression of specific genes[27,28]. Many proteins involved in DNA methylation, such as MeCP2, MBD and DNMTs, have been well characterized in human cells. However, it remains unclear how and under what circumstances they are guided to and methylate specific CpG target sites of cognate genes during the genesis or development of tumours.

In plants, long and short dsRNAs can induce sequence-specific DNA methylation, known as RNA-directed DNA methylation[3–8]. In addition, transgenes can also induce sequence-specific DNA methylation[29,30]. These phenomena might reflect the role of these systems as a cellular defence against RNA and DNA viruses. However, it remains to be determined whether similar RNA-directed DNA methylation and transgene-mediated defence systems exist in human cells.

We have demonstrated that synthetic and vector-based siRNAs can induce sequence-specific and *DNMT1/DNMT3B*-dependent RNA-mediated DNA methylation in human cells. Our siRNAs

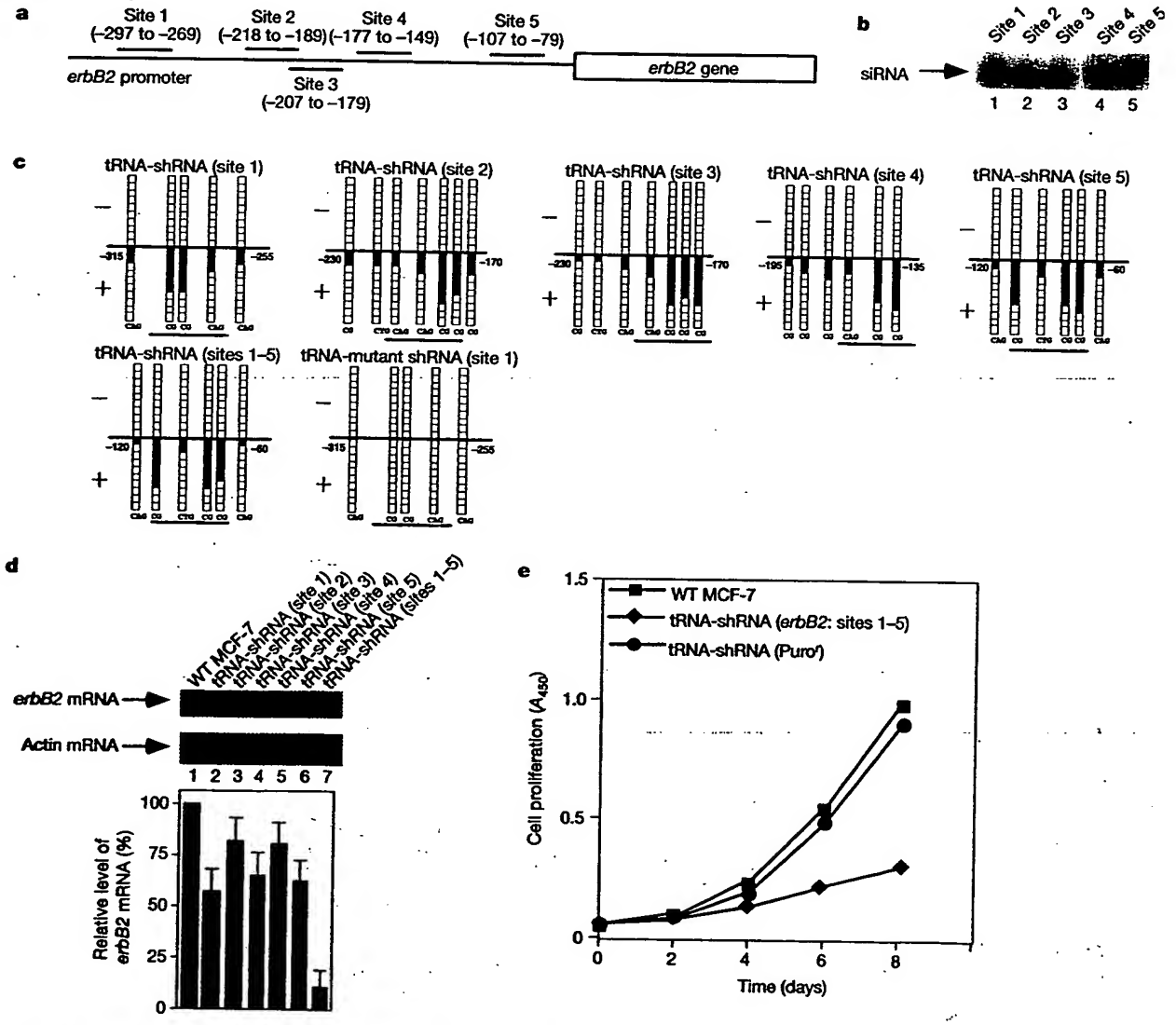

Figure 4 Induction of DNA methylation of the *erbB2* promoter by tRNA-shRNAs. **a**, Five targets for tRNA-shRNAs were selected within the *erbB2* promoter. **b**, Detection, via northern blots, of the expression of tRNA-shRNAs targeted to the *erbB2* promoter. All anticipated siRNAs were detected in cells that expressed tRNA-shRNAs. **c**, Induction by shRNAs of DNA methylation of the *erbB2* promoter, as detected by bisulphite sequencing. **d**, Relative levels of *erbB2* mRNA in cells that expressed tRNA-shRNAs targeted to the *erbB2* promoter. Total RNA from each line was analysed by northern blotting with specific probes. Values are means ± s.d. **e**, Proliferation rates of cells expressing tRNA-shRNAs. tRNA-shRNA (puro^r), targeted to a puromycin-resistance gene, was used as a control shRNA.

induced sequence-specific gene silencing at the transcriptional level. The ways in which siRNAs are guided to and gain access to genomic DNA remain unknown. Synthetic and tRNA vector-based siRNAs are localized predominantly in the cytoplasm, where siRNA-mediated degradation of mRNA also occurs. A small fraction of siRNA–protein complexes might be transported to the nucleus. Alternatively, siRNAs might gain access to genomic DNA during cell division, when the nuclear membrane disappears.

Further investigation of correlations between siRNA-induced DNA methylation and tumour genesis, anti-viral defence and epigenesis in development should provide an insight into small-RNA-induced gene silencing and development. Moreover, it is now possible to control the expression levels of specific genes in mammalian cells using siRNAs not only to disrupt cognate mRNAs but also to interfere with transcription, as demonstrated here. However, it remains to be examined precisely which promoters can be specifically methylated by respective siRNAs. Exploitation of shRNA expression vectors targeted to cognate promoters might even have potential utility in a clinical setting. □

Methods

Preparation of siRNAs

Synthetic siRNAs directed against the E-cadherin promoter (E-cadherin-siRNA) and against DMNT mRNAs (DMNT-siRNAs) were synthesized with a DNA/RNA synthesizer (model 394; PE Applied Biosystems). Sequences of E-cadherin-siRNAs are described in the Supplementary Information. For generation of siRNAs, all synthetic RNAs were annealed by the standard method[13]. We introduced siRNAs into MCF-7 cells using Oligofectamine (Invitrogen) in accordance with the manufacturer's protocol.

Construction of tRNA-shRNA expression plasmids

To construct vectors for expression of tRNA-shRNA targeted to the *erbB2* promoter, we used the pPUR-tRNA plasmid, which includes a chemically synthesized promoter for a human gene for $tRNA^{Val}$ between the *Eco*RI and *Bam*HI sites of pPUR (Clontech)[16]. Sequences (sites 1–5) of shRNAs targeted to the *erbB2* promoter are described in Supplementary Information. Chemically synthesized oligonucleotides encoding *erbB2* promoter-directed shRNAs that included a loop motif were amplified as double-stranded sequences by PCR. After digestion with *Sac*I and *Kpn*I, the fragments were cloned downstream of the promoter of the $tRNA^{Val}$ gene in pPUR-tRNA.

Bisulphite sequencing of E-cadherin and *erbB2* promoters

We extracted genomic DNA from cells by standard methods using proteinase K, phenol and chloroform. We performed bisulphite modifications using a CpGenome DNA modification kit (Intergen) following the manufacturer's instructions. We amplified the bisulphite-modified E-cadherin and *erbB2* promoters using specific primers as follows: for the E-cadherin promoter, forward primer 5′-TCTAGAAAAATTTTTTAAAAA-3′ and reverse primer 5′-CAGCGCCGAGAGGCTGCGGCT-3′; for the *erb*B2 promoter, forward primer 5′-CCTGGAAGCCACAAGGTAAAC-3′ and reverse primer 5′-TTTCTCCGGTCCCAATGGAGG-3′. The amplified DNAs were subcloned into the TA-cloning vector. Then we picked ten independent colonies in each case, determined the sequence of the promoter in each plasmid and examined the extent of methylation by determining the number and position of methylated cytosine residues.

Culture and transfection of cells

Human MCF-7 cells were cultured in minimum essential medium (MEM) supplemented with 10% FCS, 1% NEAA and 1 mM Na-Pyr. Human normal mammary epithelial cells (CAMBREX) were grown with mammary epithelial cell medium (CAMBREX). Transfection with pPUR-tRNA-shRNA was performed with the Effectene reagent (Qiagen) according to the manufacturer's protocol. For transfection of MCF-7 cells with various siRNAs, we used Oligfectamine (Invitrogen) according to the manufacturer's protocol.

5-aza-2′-deoxycytidine treatment

For inhibition of DNA methylation by 5-aza-2′-deoxycytidine (5-aza), cells grown to 30–40% confluence were transfected with E-cadherin-siRNAs and then were incubated for 96 h in culture medium that contained a final concentration of 1 μM 5-aza (Sigma).

Northern blotting analysis

Total RNA was purified from MCF-7 cells that expressed tRNA-shRNAs targeted to the *erbB2* promoter with ISOGEN reagent (Wako). Thirty micrograms of total RNA per lane were loaded on a 15% polyacrylamide gel. After electrophoresis, bands of RNA were transferred to a Hybond-N nylon membrane (Amersham). The RNA on the membrane was allowed to hybridize to ^{32}P-labelled probes that were complementary to the sequences of the tRNA-shRNAs. Sequences of synthetic probes were as follows: site 1, 5′-CUCUG CCCCCUCCCCCGGAGUCCGGGAUAA-3′; site 2, 5′-UCCUAGCGCCGGGAAGCU GGGUUGCCUGCA-3′; site 3, 5′-GGUGCGUCCCUCCUAGCGCCGGGAAGCUGG-3′; site 4, 5′-GGUGCGUCCCUCCUAGCGCCGGGAAGCUGG-3′; and site 5, 5′-GAGCAA GCGCGCUCCCAGCUCGCCCCCUCC-3′. For detection of the expression level of E-cadherin, *erbB2* and actin mRNAs, we used cDNA probes of E-cadherin, *erbB2* and actin, respectively.

Western blotting analysis

MCF-7 cells transfected with or without various E-cadherin-siRNAs targeted to the E-cadherin promoter and various tRNA-shRNAs targeted to the *erbB2* promoter were collected. Total protein (20 μg) was fractionated by SDS–polyacrylamide gel electrophoresis (10% polyacrylamide) and bands of protein were transferred to a polyvinylidene difluoride membrane (Funakoshi) by electro-blotting. Immunocomplexes were visualized with an ECL kit (Amersham) after reactions with monoclonal antibodies against E-cadherin (Transduction laboratories), ErbB2 (Oncogene), actin (Chemicon), DNMT1 (Imgenex) and DNMT3B (Imgenex), or with polyclonal antibodies against DNMT2 (Imgenex). Amounts of E-cadherin and ErbB2 were normalized by reference amounts of actin.

Determination of cell proliferation rates

Cell proliferation rates were determined with a Cell Proliferation Kit II (Roche) according to the manufacturer's instructions[16].

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Correspondence and requests for materials should be addressed to K.T. (taira@chembio.t.u-tokyo.ac.jp) or H.K. (kawasaki@chembio.t.u-tokyo.ac.jp).

Error-prone replication of oxidatively damaged DNA by a high-fidelity DNA polymerase

Gerald W. Hsu[1], Matthias Ober[2], Thomas Carell[2] & Lorena S. Beese[1]

[1]*Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710, USA*
[2]*Department of Chemistry and Biochemistry, Ludwig Maximilians University Munich, Butenandtstrasse 5-13, D 81377 Munich, Germany*

Aerobic respiration generates reactive oxygen species that can damage guanine residues and lead to the production of 8-oxoguanine (8oxoG), the major mutagenic oxidative lesion in the genome[1]. Oxidative damage is implicated in ageing[2] and cancer, and its prevalence presents a constant challenge to DNA polymerases that ensure accurate transmission of genomic information. When these polymerases encounter 8oxoG, they frequently catalyse misincorporation of adenine in preference to accurate incorporation of cytosine[3]. This results in the propagation of G to T transversions, which are commonly observed somatic mutations associated with human cancers[4,5]. Here, we present sequential snapshots of a high-fidelity DNA polymerase during both accurate and mutagenic replication of 8oxoG. Comparison of these crystal structures reveals that 8oxoG induces an inversion of the mismatch recognition mechanisms that normally proofread DNA, such that the 8oxoG·adenine mismatch mimics a cognate base pair whereas the 8oxoG·cytosine base pair behaves as a mismatch. These studies reveal a fundamental mechanism of error-prone replication and show how 8oxoG, and DNA lesions in general, can form mismatches that evade polymerase error-detection mechanisms, potentially leading to the stable incorporation of lethal mutations.

Many DNA damage lesions stall or block DNA replication; however, 8oxoG is bypassed efficiently and inaccurately by high-fidelity polymerases[3,6–8]. 8oxoG retains the ability to engage in correct Watson–Crick base pairs with C, but oxidation of G (at C8) converts a hydrogen bond acceptor (N7) to a hydrogen bond donor, allowing a stable Hoogsteen base pair to form between 8oxoG and A, which is not possible in undamaged DNA (Fig. 1). In the absence of accessory factors such as proliferating cell nuclear antigen (PCNA), preferential mutagenic translesion replication of 8oxoG by the major replicative DNA polymerases α and δ *in vitro* indicates that 8oxoG is not recognized as a DNA lesion. Instead, the A·8oxoG mismatch evades the intrinsic mechanisms that cause DNA polymerases to achieve high-fidelity replication of undamaged DNA.

Insights into the mechanisms of faithful replication by high-fidelity polymerases[9] and mismatch recognition[10] have been provided by capturing structures of the replication cycle of the DNA polymerase I fragment from a thermostable strain of *Bacillus stearothermophilus* (BF) in a polymerase crystal that retains the ability to replicate DNA[11]. Throughout the replicative cycle of BF, there are many points at which recognition of DNA mismatches or lesions can occur. First, during the transition of the template strand from a pre-insertion site that sequesters the template base before nucleotide incorporation to an insertion site where the template base interacts with the incoming nucleotide, the conformations of the template base and the incoming nucleotide are both tightly regulated to ensure that hydrogen bonding interactions are limited to the Watson–Crick faces of the nascent base pair. Second, before covalent incorporation of the paired dNTP at the insertion site, the polymerase selects for base pairs that exhibit the shape and geometry of cognate Watson–Crick base pairs in preference to ones that do not[12,13]. Third, after covalent incorporation, the new base pair moves to a post-insertion site where residues Arg 615 and Gln 797 form hydrogen bonds to the DNA minor groove of correctly formed base pairs. At this step, DNA mismatches induce distortions within the polymerase active site that cause the polymerase to stall and dissociate[10]. Last, covalently incorporated mismatches or lesions continue to impede replication from up to four base pairs away[14], inducing distortions to the active site as they translocate along the surface of the polymerase[10]. In this way, a molecular 'memory' of the mismatch is retained by the polymerase.

To investigate whether BF behaves analogously to replicative polymerases α and δ with respect to replication of 8oxoG, primer extension assays were performed (Fig. 2) and steady-state kinetic parameters were determined for incorporation of dCTP or dATP opposite 8oxoG (Table 1). Comparison of the specificity constants (k_{cat}/K_m; where k_{cat} is the turnover number and K_m is the Michaelis constant) for incorporation of these nucleotides opposite 8oxoG indicates that misincorporation of dATP is ninefold more efficient than dCTP incorporation, whereas in undamaged DNA, accurate dCTP incorporation opposite an unmodified guanine is 10^6-fold more efficient than misincorporation of dATP. After incorporation of either dATP or dCTP, extension past the newly formed 8oxoG base pair is readily observed (Fig. 2a, b) and proceeds to the end of the template in the presence of a full complement of dNTPs (Supplementary Fig. 1). These results indicate that BF, as with replicative polymerases α and δ, preferentially misincorporates dATP opposite 8oxoG. BF can therefore serve as a model system to address mutagenic 8oxoG replication.

To determine the effects of 8oxoG on the structural mechanisms

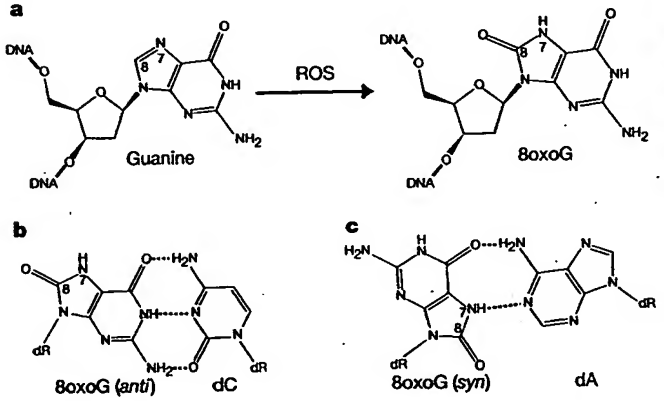

Figure 1 Modes of base pairing for 8oxoG. **a**, Oxidation of guanine at C8 by reactive oxygen species (ROS). **b**, 8oxoG in a Watson–Crick base pair with dC. Dashed lines indicate potential hydrogen bonds. **c**, 8oxoG (*syn*) in a Hoogsteen base pair with dA (*anti*).